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Binding–gating coupling in a nondesensitizing α7 nicotinic receptor A single channel pharmacological study

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ABSTRACT

The highly conserved αLys145 has been suggested to play an important role in the early steps of activation of the nicotinic acetylcholine receptor (nAChR) by acetylcholine. Both macroscopic and single channel currents were recorded in the slowly desensitizing mutants *L248T*- and *K145A-L248T-α7* receptors expressed in *Xenopus* oocytes. On ACh-evoked currents, substitution of Lys145 by alanine showed the same effects that in wild type receptors: moderately decreased gating function and a more-than-expected loss of ACh potency, thus validating the experimental model. Single channel analysis quantitatively agreed with macroscopic data and revealed that impaired gating function in the double mutant *α7K145A/L248T* is the consequence of a slower opening rate, *β*. Several nicotinic agonists were also studied, showing important features. Particularly, dimethylphenylpiperazinium (DMPP), acting as an antagonist in α7K145A, became a full agonist in α7K145A/L248T. Single channel analysis of DMPP-evoked currents showed effects of Lys145 removal similar to those observed with ACh. Data suggest that α7Lys145 facilitates the early steps of channel activation. Moreover, the slowly desensitizing mutant *α7L248T* could be an interesting tool for the study of channel activation in α7 receptors. Nevertheless, its extensively altered pharmacology precludes the simple extrapolation of pharmacological data obtained in singly mutated α7 receptors.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) mediate fast synaptic transmission in muscle and nerve cells. They are members of the Cys-loop family of ligand-gated ion channels, which includes 5-HT_3, γ-$GABA_A$ receptors, and glycine receptors [1,2]. Such receptors are allosteric proteins because the signal generated at the binding-site region must be transmitted to the channel gate located some distance away, and this process involves a more than local conformational change [3]. Recent structural data from the *Torpedo* nAChR [4,5] and from the acetylcholine binding protein (AChBP) of snails [6,7] have been helping to understand the coupling between binding and gating in Cys-loop receptors. However, the precise molecular mechanisms involved in channel activation are still poorly understood [8]. According to the current model of activation, the interaction of the agonist with the binding site would generate a motion of the α subunits that is transmitted to the gate through rearrangements of several extracellular structures. Among them, the highly conserved Lys145, at the β-strand β7 of nAChR α subunits, is located close to both the Cys-loop and the binding segment C, the latter containing several aromatic residues involved in binding of nicotinic agonists and antagonists [6,9]. Such a unique position and its possible role in binding–gating coupling had been pointed out after analysis of crystallographic data obtained in the snail AChBP [10]. This role was confirmed in both muscle- and neuronal-type α7 nAChRs [11,12]. In addition to its role in coupling, it has been reported that Lys145 also determines the pharmacological profile of homomeric α7 nAChRs because DMPP can become a competitive antagonist after mutating Lys145 [12].

However, neuronal type α7 nAChRs currents show fast desensitization which precludes the use of single channel data to study the mechanisms altered by mutations in Lys145 more precisely. The alternative used in the present work is to include such mutations in a slowly- or non-desensitizing α7 nAChR, as it is α7L248T nAChR. In such receptors, a highly conserved leucine residue of the pore domain has been substituted by a threonine, and the resulting receptors did not show the typical fast desensitization of homomeric α7 nAChR [13], and also presented an unconventional pharmacology [14–17]. This non-desensitizing receptor has also been helpful in establishing the role of several residues both in coupling and calcium modulation of α7 nAChR [18,19].

In the present work, we have further studied the role of Lys145 in binding–gating coupling of neuronal type α7 nAChRs by including the mutation K145A in α7L248T nAChR, and recording agonist-activated single channel currents. Our data show that impairment of coupling was mostly due to a delay in the channel activation. Moreover, α7L248T nAChRs has been proven to be a useful tool for studying at

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the single channel level mutations affecting ACh activation of α7 nAChRs. However, the pharmacological profile of single mutant *α7K145A* could not be transferred to α7L248T nAChRs.

2. Materials and methods

2.1. Generation of mutants of the bovine α7 subunit

The bovine α7 cDNA was cloned in a derivative of the pSP64T vector [20] containing part of the pBluescript polylinker. To generate the mutants we annealed single-stranded oligonucleotides with the desired sequences and proper single-strand ends that could be easily ligated to the ends generated through restriction enzymes digestion of the α7 cDNA. The restriction enzyme sites for cloning the annealed oligonucleotides were either present in the original cDNA sequence or introduced as silent mutations [21] by using mutated oligonucleotides and PCR (25 cycles at 94 °C for 10 s, 60 °C for 30 s, 72 °C for 45 s).

2.2. Oocyte expression

Capped mRNA was synthesized *in vitro* using SP6 RNA polymerase, the mMESSAGE mMACHINE kit (Ambion, Texas) and the pSP64T derivative mentioned above. Defoliculated *Xenopus laevis* oocytes were injected either with 2.5 (α7K145A/L248T) or 0.5 (α7L248T) ng of total cRNA in 50 nl of sterile water. Oocytes were incubated in calcium-free medium containing 1 μM methyllycaconitine (MLA). All experiments were performed within 3–4 days after cRNA injection.

2.3. ^{125}I-α-bungarotoxin binding assays

Specific surface expression of ^{125}I-α-bungarotoxin (α-Bgt) binding sites was tested with 10 nM ^{125}I-α-Bgt as described [22]. Briefly, oocytes located in 24-well plates were preincubated for 15 min with Barth's buffer containing 5% of fetal calf serum and further incubated in the same medium with 10 nM ^{125}I-α-Bgt for 2 h at 18 °C in a final volume of 300 μl. At the end of the incubation, unbound ^{125}I-α-Bgt was removed, oocytes were passed to 6-well plates, washed five times with 4 ml Barth's buffer and bound radioactivity was counted. Dissociation constants of ^{125}I-α-Bgt were 3.8 and 2.9 nM for α7L248T and α7K145/L248T, respectively (not significant) and, therefore, the errors derived from not using a saturating concentration should be small. Nonspecific binding was determined using uninjected oocytes.

2.4. Electrophysiological recordings

Electrophysiological recordings of whole-cell currents were done as described previously [22]. Some oocytes showed holding currents which were mostly blocked by 1 μM MLA suggesting spontaneous activity of nAChRs. In order to simplify the analysis, oocytes showing holding currents >1% of the maximal ACh-evoked currents were discarded. Extracellular solution contained (in mM): NaCl 82.5, KCl 2.5, $BaCl_2$ 2.5, $MgCl_2$ 1 and HEPES 5 (pH 7.4). The velocity of application of agonists was 18–22 ml min^{-1}. All experiments were done at room temperature (22 °C). Unless otherwise indicated, oocyte membrane potential was held at −40 mV. Data analysis was performed with the software package Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Dose–response curves were fitted using a nonlinear least squares algorithm to the Hill equation, $I/I_{max} = 1/(1+(EC_{50}/C)^h)$, were EC_{50} is the agonist concentration that elicits the half-maximal response, h is the Hill coefficient, and C is the agonist concentration.

Single channel records were obtained in the cell-attached mode using an Axopatch 200A amplifier (Axon Instruments, CA). Oocytes were stripped of their vitelline membrane by immersing them in a hypertonic solution to shrink them enough so that the vitelline membrane was visible and then could be removed with thin tip tweezers. They were then transferred to a recording chamber with a modified extracellular solution where NaCl was substituted by KCl to clamp the membrane potential close to 0 mV [23]. Holding potential was −80 mV. Patch pipettes were pulled from thick-walled borosilicate glass (GC150-15, Clark Electromedical Instruments), with a resistance between 5 and 10 MΩ when filled with standard extracellular solution. ACh or DMPP were added to the pipette solution. Initially 10 μM gadolinium was added to the pipette to block stretch activated channels [24], but in later experiments gadolinium was not used because stretch activated channels were not observed if the negative pressure was released after making the seal and, furthermore, nicotine activated receptors could be recognized by their kinetics (long openings) and conductance range (with large and uniform currents). Records were low-pass filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices, Inc.), sampled at 20 kHz and stored on hard disk for later analysis.

2.5. Data analysis

Single channel recordings were analyzed with the Clampfit 9 (Molecular Devices) and the QUB software suite (version 1.4.0.2) [25]. For each patch a voltage pulse protocol was applied to determine the conductance (γ) and reversal potential (V_r) of the channels. Ten second duration pulses were applied from +60 to −120 mV at 20 mV intervals. Single channel current–voltage curves were fitted with Prism 4 (GraphPad Software, Inc.) to the equation $I=\gamma\,(V-V_r)$. Open probabilities were estimated from all point histograms fitted to a sum of Gaussians with Clampfit routines.

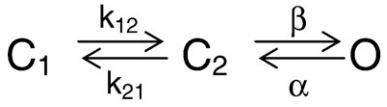
Kinetic analysis was performed using QUB on records with a dead time of 0.1 ms, idealized by the half amplitude threshold method, and restricted to bursts of activity of a single channel. Bursts were identified by a closed interval longer than a given burst terminator time that usually was between 10 and 30 ms, and containing more than 3 opening events. The selected region of the idealized data, obtained as described above, was then fitted to a simple linear three-state model with two closed and one open state, with four kinetic constants, as shown in kinetic Scheme 1, with the MIL method that gives directly an estimate of the kinetic constants their standard errors. Dwell time histograms with the corresponding time constants, and their relative weight, were also obtained with the same program.

Data are presented as mean±standard error. Statistical significance was calculated by non-paired Student's t-test. EC_{50} values were compared with an F test. The null hypothesis was rejected when $p<0.05$.

3. Results

3.1. Effect of the mutation K145A on the functional responses to ACh in the slowly desensitizing L248T receptor

Fig. 1A shows two families of macroscopic inward ACh-evoked currents in the single (α7L248T) and double (*α7K145A/L248T*) mutant receptors, respectively. Inward currents evoked at equiactive ACh concentrations presented the same kinetics both in α7L248T and α7K145A/L248T receptors. Maximal currents in α7K145A/L248T were almost 2-fold larger than in α7L248T. However, surface expression, as measured by α-bungarotoxin (α-Bgt) binding, was also increased around 4-fold. α7L248T and α7K145A/L248T oocytes expressed 0.5±0.1 and 1.8±0.4 fmol of α-Bgt, respectively (n=4). Nevertheless, such a difference does not point to a more efficient surface expression of α7K145A/L248T receptors because of the differences in the amount of



Scheme 1.

cRNA injected (see Methods). After normalizing functional responses to surface expression, α7K145A/L248T receptors showed a gating function of 41±12% of α7248T receptors (n=4). This reduction in gating function is close to that obtained after the inclusion of the same mutation in wild type α7 receptors [12]. Concentration–responses relationship in α7L248T receptors showed the typical reduction in the EC_{50} value with respect to wild type receptors (around 60-fold lower). However, α7K145A/L248T receptors showed a shift in ACh potency similar to that observed when starting with α7 wild type receptors, because EC_{50} value increased from 0.65 to 15.7 μM (Fig. 1B). As occurred in wild type receptors, this change in the EC_{50} value is not quantitatively predicted by only considering the modification in gating function. Thus, a change in the ACh binding properties is likely to have taken place as well.

We have also explored the possible functional interaction between residues located at positions of Lys145 and Leu248 by calculating the coupling coefficient Ω of the two-mutant cycle [12,26,27]. Ω values were 0.42 and 0.57 for the current–binding ratio and for the EC_{50}, respectively, suggesting that both positions are only weakly coupled, and thus, effects of mutating Lys145 are not only qualitatively, but also quantitatively similar in both α7 wild type and α7L248T receptors.

3.2. Acetylcholine-evoked unitary currents in single and double mutants

So far, substitution of Lys145 by alanine showed the same effects in α7L248T than in α7 wild type receptors. The slowly desensitizing properties of single mutant *α7L248T* allowed the acquisition of single channel recordings in the cell-attached configuration of the patch-clamp technique. In the absence of agonist α7L248T receptors showed very brief spontaneous openings. However, when the oocyte membrane is exposed to a high ACh concentration (100 μM) large and frequent unitary inward currents of about 12 pA are observed with both receptors (Fig. 2). Differences in single channel conductances were small but significant (105±3 and 114±2 pS in α7L248T and α7K145A/L248T receptors, respectively). However, these results discard that reduced gating function in α7K145A/L248T was due to a decrease in the unitary conductance. Very brief events with a smaller conductance could also be seen in both receptors, but they contribute very little to the overall probability and were not included in the dwell time analysis. α7K145A/L248T receptors show both longer closings and somewhat shorter openings than α7L248T receptors. Single channels showed open probabilities of 0.17 and 0.06 in α7L248T and α7K145A/L248T receptors, respectively. Those probabilities were estimated from fitting all-points histograms with Gaussian functions and comparing the areas. The estimated ratio of open channel probabilities (35% of α7L248T receptors) is close to that obtained previously from macroscopic currents.

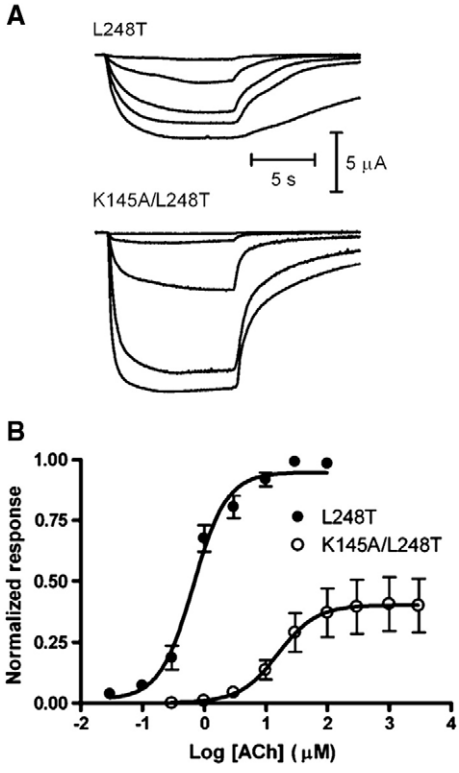


Fig. 1. A comparison between the ACh responses of α7L248T nAChR and double mutant *α7K145A/L248T*. (A) Inward currents obtained upon stimulation with ACh of α7L248T (top, control) and α7K145A/L248T (bottom) nAChRs. Traces represent currents obtained at −40 mV. For each family of currents, ACh concentrations were (from top to bottom): 0.1, 0.3, 1, 3 and 10 μM (α7L248T), and 1, 3, 10, 30 and 100 μM (α7K145A/L248T). (B) ACh concentration–response curves of oocytes expressing αL248T (*filled circles*) and α7K145A/L248T receptors (*open circles*). Data are normalized to the estimated maximal response to ACh (both receptors) and corrected for different surface expression (α7K145A/L248T receptors, error included). Data points represent means±SEM obtained in 8–16 oocytes from 3–4 donors. Error bars shown if bigger than symbols. Continuous lines represent fits of data to the Hill equation. α7L248T: EC_{50}=0.65±0.07 μM, h=1.51. α7K145A/L248T: EC_{50}=15.6±1.3 μM, h=1.36.

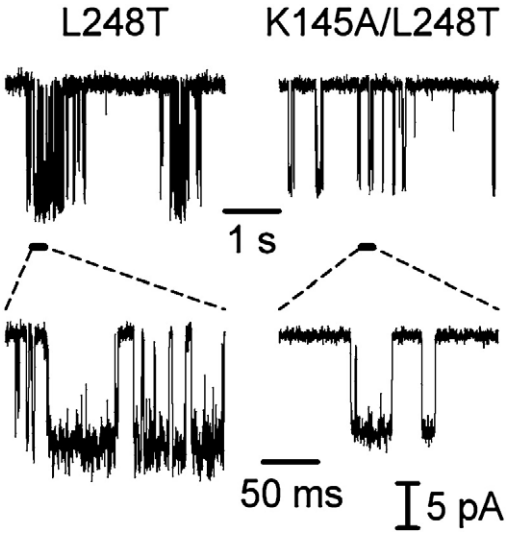


Fig. 2. Acetylcholine-evoked unitary currents. Top panels: Representative traces of single channel currents evoked by 100 μM ACh in α7L248T and α7K145A/L248T receptors. Bottom panels: Expanded scale representations of the horizontal bars in top panels. Holding potential was −100 mV.

Dwell time analysis was also carried out in α7L248T and α7K145A/L248T receptors. Fig. 3 shows the analysis of two representative membrane patches. These data could be analyzed in terms of a given kinetic model. For the sake of simplicity, a simple linear three-state kinetic model (two closed states and one open state; Scheme 1) was applied to data, and open time distributions were fitted with mean open times of 28 and 19 ms, for α7L248T and α7K145A/L248T receptors, respectively (Table 1). Such a difference was not statistically significant. Accordingly with Scheme 1, closed times showed a two-exponential distribution with mean closed times of 1.0 (weight: 0.78), and 31 ms in α7L248T receptors, with a clear dominance of the shortest closures. When the same analysis is performed in α7K145A/L248T receptors, mean closed times were longer (1.6 (weight: 0.45), and 62 ms), and longer closures were much more frequent (Table 1).

Values for the several first order kinetic rates could be estimated as well (Table 2). Such analysis revealed that the reduction in open probability in α7K145A/L248T receptors is mostly attributed to an impairment of channel opening due to statistically significant decrease in the opening rate constant, β, being all other rate constants not statistically different from α7L248T receptors. Interestingly, the gating constant, β/α, in α7K145A/L248T receptors was 30% of α7L248T

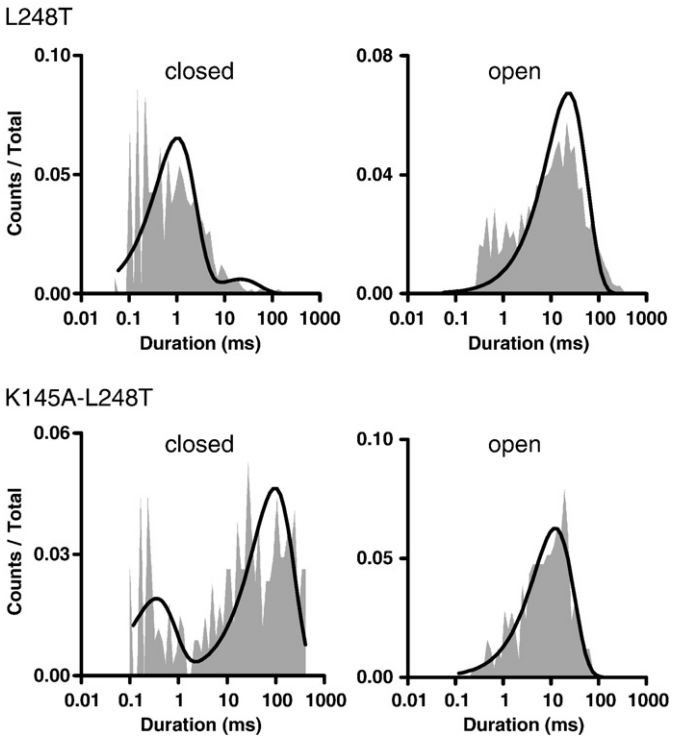


Fig. 3. Dwell-time histograms of acetylcholine-evoked single channel events. Closed (left) and open (right) time histograms for α7/L248T (top) and α7K145A/L248T (bottom) ACh-evoked unitary currents. Logarithmic binning and linear scale for relative frequency of events were used. Superimposed continuous lines are fits for Scheme 1. Holding potential was −100 mV.

receptors, indicating that the changes in these gating rates quantitatively account for most of the reduction in overall currents. Taken together, data suggest that the reduction of whole-cell currents in α7K145A/L248T receptors is mainly caused by impairment in the channel activation rather than a destabilization of the open configuration.

3.3. Pharmacological profile

Several mutations of Lys145 in wild type receptors induced changes in the pharmacological profile of the resulting receptors [12]. Therefore, we checked the functional responses of α7L248T and α7K145A/L248T receptors to several nicotinic drugs. All additional agonists tested evoked ionic currents that were kinetically indistinguishable from ACh-evoked currents both in α7L248T and in α7K145A/L248T receptors (not shown). Fig. 4 shows concentration–response relationship for nicotine, epibatidine, cytisine and DMPP. When compared to α7L248T curves, those obtained with α7K145A/L248T receptors were consistently shifted to the right, showing statistically significant higher EC_{50} values. When the same mutation was inserted in wild type α7 receptors, there were also changes in the agonist potency, but they were small except for ACh. However, the shifts observed in the present work were not always of the same

Table 1
Time constants extracted from the dwell-time distributions

Agonist	Receptor	τ_{c1} (weight)	τ_{c2}	τ_o	N
Ach	α7L248T	0.97±0.13 (0.78±0.08)	31.5±5.9	28.0±4.6	6
	α7K145A/L248T	1.62±0.52 (0.45±0.08)	62.4±12.8	18.5±4.9	8
DMPP	α7L248T	0.75±0.23 (0.55±0.14)	19.9±2.4	1.94±0.49	5
	α7K145A/L248T	1.51±0.25 (0.76±0.05)	15.5±2.7	1.05±0.24	6

Agonist concentrations used were 100 and 10 μM for ACh and DMPP, respectively. Time constants are in ms. *N* stands for the number of patches analyzed.

Table 2
Kinetic parameters estimated from a fit to Scheme 1

Agonist	Receptor	K_{12}	K_{21}	β	α	β/α
Ach	α7L248T	55±17	274±109	863±92	43±7	24±5
	α7K145A/L248T	54±14	730±272	474±96*	86±21	7.3±1.8*
DMPP	α7L248T	119±42	1081±642	1168±177	813±294	1.8±0.5
	α7K145A/L248T	68±3	172±75	692±87*	1129±251	0.6±0.1*

Data analyzed and agonist concentrations are as in Table 1. Rate constants are in s^{-1}.
* Statistically different from α7L248T ($p<0.05$).

magnitude but strongly depended on the agonist used. The loss of potency in α7K145A/L248T receptors ranged from 1.8-fold with epibatidine up to 125-fold with cytisine. Nicotine and DMPP showed in-between values, similar to those obtained with ACh (Fig. 1). When maximal currents were considered, all tested agonists showed the same extent of functional response when compared to ACh in the same receptor (Fig. 5). Only cytisine in α7L248T receptors showed a small but statistically significant reduction in maximal currents. This is in great contrast to results obtained with α7 wild type and α7K145A receptors [12]. In α7 wild type receptors, all five agonists evoked similar responses and, when Lys145 was substituted by Ala, responses to epibatidine and cytisine were only moderately reduced, ACh and nicotine were significantly less efficacious, and DMPP became a competitive antagonist. The same behavior of DMPP was observed with carbamylcholine, which was ineffective in the single mutant *α7K145A*, but evoked full responses in the double mutant *α7K145A/L248T* (not shown).

Competitive nicotinic antagonists and other non-nicotinic drugs have been reported to activate α7/L248T receptors. Thus, we checked whether the double mutant *α7K145A/L248T* retained these unusual

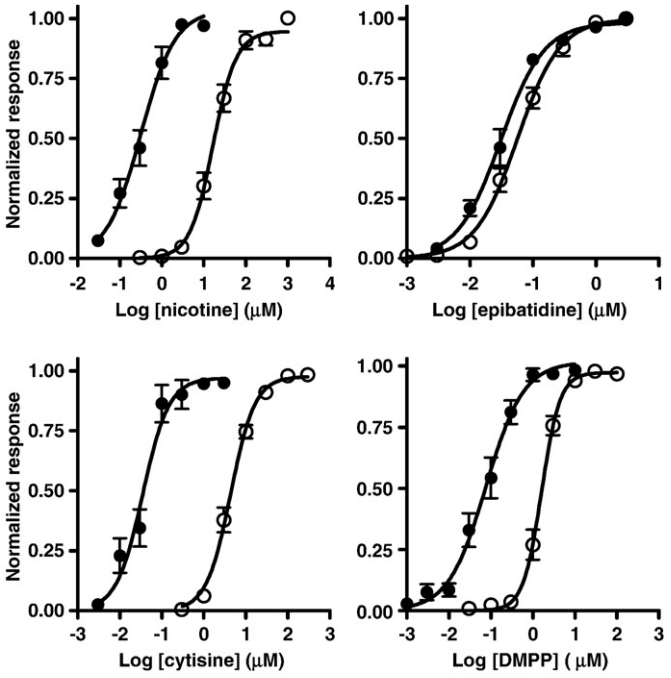


Fig. 4. Concentration–response relationships. Agonist concentration–response curves of oocytes expressing α7L248T (*filled circles*) and α7K145A/L248T (*open circles*) nAChRs for nicotine, epibatidine, cytisine and DMPP. For each receptor and agonist, data are normalized to their maximal response. Data points represent means±SEM obtained in 4–7 oocytes from 3 donors. Error bars shown if bigger than symbols. Continuous lines represent fits of data to the Hill equation. Nicotine=0.32±0.07 μM, h=1.06; Nicotine—α7K145A/L248T: EC_{50}=16.7±1.6 μM, h=1.58. Epibatidine—α7L248T: EC_{50}=0.031±0.004 μM, h=1.27; Epibatidine—α7K145A/L248T: EC_{50}=0.057±0.006 μM, h=1.29. Cytisine—α7L248T: EC_{50}=0.035±0.007 μM, h=1.39; Cytisine—α7K145A/L248T: EC_{50}=4.38±0.29 μM, h=1.51. DMPP—α7L248T: EC_{50}=0.077±0.016 μM, h=0.97; DMPP—α7K145A/L248T: EC_{50}=1.61±0.11 μM, h=1.98.

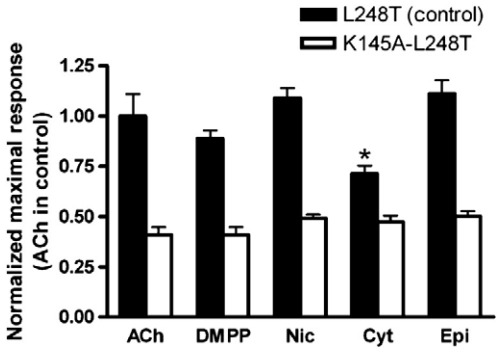


Fig. 5. Maximal currents. Mean maximal currents ± SEM upon stimulation with nicotinic agonists in α7L248T (*filled bars*) and α7K145A/L248T receptors (*open bars*). Inward currents were obtained at −40 mV with saturating concentrations of each agonist, and normalized to the maximal response achieved by ACh in the same oocyte. Data of α7K145A/L248T receptors were corrected for different surface expression of receptors.

pharmacological features. Dihydro-β-erythroidin, d-tubocurarine, strychnine and serotonin, all of them were capable to elicit agonist responses in this double mutant (not shown).

3.4. DMPP-evoked unitary currents in single and double mutants

Since DMPP was able to activate nicotinic currents in α7K145A/L248T receptors to the same extent than ACh, DMPP-evoked single channel currents were recorded in the cell-attached configuration (Fig. 6). Unitary conductances were similar to those observed with ACh. For DMPP-evoked currents, they were estimated in 102 ± 9 and 114 ± 13 pS, in α7L248T and α7K145A/L248T receptors, respectively (not significant difference). Open probabilities were estimated to be 0.16 and 0.07, very close to those of ACh and in good agreement with macroscopic data. However, both α7L248T and α7K145A/L248T receptors also showed several significant differences regarding the activating agonist. First of all, DMPP-evoked openings are much shorter and, besides, a subconductance state makes a clear contribution to the total current (Fig. 6).

Dwell time distributions were also analyzed (Fig. 7). According to Scheme 1, DMPP-activated channels in α7L248T receptors showed mean open times of 1.9 ms (Table 1), more than one order of magnitude shorter than with ACh. Closed times were also briefer (0.8 (weight: 0.55), and 20 ms), but the difference with ACh was not large. However, in α7K145A/L248T receptors, DMPP evoked slightly shorter openings (mean open time of 1.1 ms, not significant difference), but the brief closures were longer and more abundant than in α7L248T receptors (mean closed times of 1.5 (weight: 0.76), and 16 ms). Thus, the effects of mutating Lys145 to Ala in α7L248T are similar for both agonists. This could also be observed in the estimates for the first-order rate constants shown in Table 2. Similarly to ACh, opening rate constant, β, was significantly slower in α7K145A/L248T receptors. However, gating constant, β/α, obtained with DMPP in α7K145A/L248T receptors was 33% of α7L248T receptors, quantitatively accounting for the reduction in open channel probability.

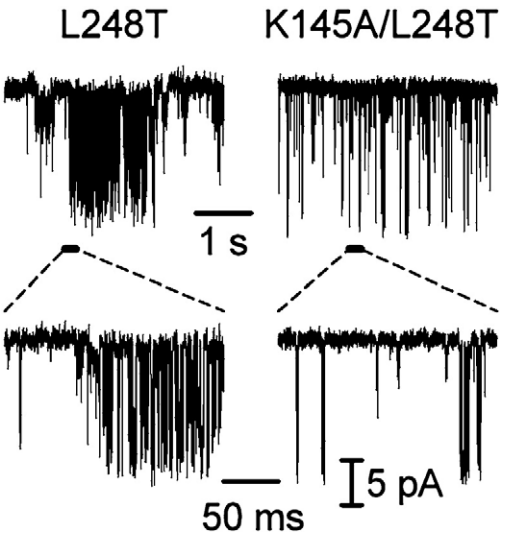


Fig. 6. DMPP-evoked unitary currents. Top panels: Representative traces of single channel currents evoked by 10 μM DMPP in α7L248T and α7K145A/L248T receptors. Bottom panels: Expanded scale representations of the horizontal bars in top panels. Holding potential was −100 mV.

4. Discussion

When ACh was used as the activating molecule, the effects of mutation K145A in α7L248T receptors were similar to those observed in wild type α7 nAChRs [12], i.e.: a decrease in gating function (estimated by the current-to-binding ratio) accompanied by a shift to the right in the dose–response curve which cannot be quantitatively accounted for by the reduced gating function [28]. Single channel events were recorded in the presence of nicotinic agonists showing a unitary conductance around 110 pS, which roughly doubles those reported for chicken α7-L247T [17] and human α7-L248T receptors [29]. The higher conductance observed here is likely to be due to a different gating mode found in bovine α7L248T receptors, rather than to the simultaneous activation of two lower conductance channels, because of two reasons: 1st) microscopic kinetics are slower [29], and 2nd) not half-conductance events were recorded.

ACh was applied at saturating concentrations and α7K145A/L248T receptors showed slightly shorter openings and considerably longer closings, when compared α7L248T receptors. When studied under a

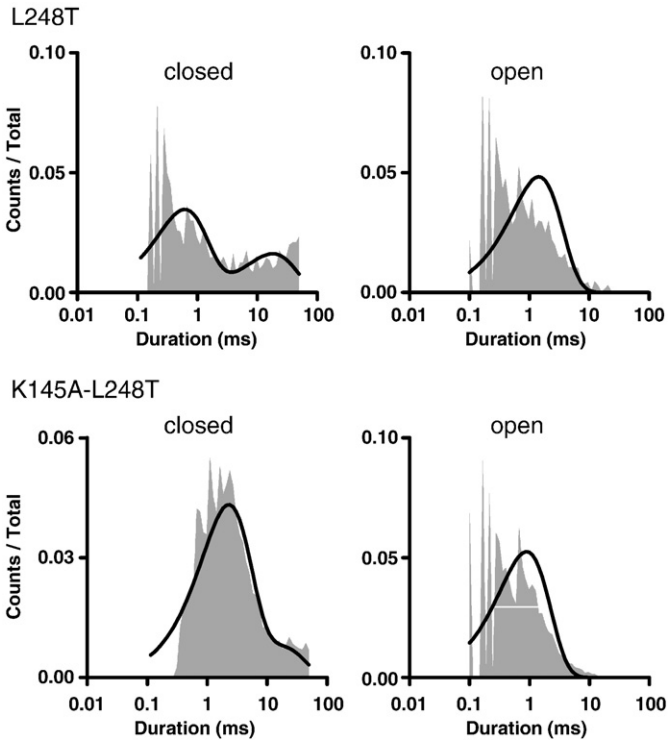


Fig. 7. Dwell-time histograms of DMPP-evoked single channel events. Closed (left) and open (right) time histograms for α7/L248T (top) and α7K145A/L248T receptors (bottom) DMPP-evoked unitary currents. Logarithmic binning and linear scale for relative frequency of events were used. Superimposed continuous lines are fits for Scheme 1. Holding potential was −100 mV.

simple kinetic scheme (two closed and one open states), those data resulted in changes of the opening rate constant (β).These results are in qualitative agreement with those obtained in muscle nAChRs showing that substitution of Ala for αK145 intensely affected receptor activation mostly by a large reduction in opening rate constant [11]. Thus, data confirm that Lys145 is involved in the early steps of muscle nAChR activation, as could have been advanced by the strategic position of Lys145. However, the effects of mutating Lys145 in neuronal-type α7L248T receptors are more modest, which is not surprising because both quantitative and qualitative discrepancies between muscle- and neuronal-type nAChR gating have been reported [12,21,27,30,31].

Several residues and/or regions of the extracellular domain of neuronal-type subunits have been identified as determinants of pharmacological selectivity in nAChRs [32–37]. The pharmacological profile of α7 AChRs is also determined by the residue present at position 145. Thus, single mutant *α7K145A* showed a great influence in the effectiveness of some nicotinic agonists (ACh, nicotine, carbamylcholine and DMPP), but not others (cytisine, epibatidine), being the most remarkable changes those of carbamylcholine and DMPP, which became competitive antagonists in mutant *α7K145A* [12]. We have checked the effectiveness and potency of those nicotinic agonists in both α7L248T and α7K145A-L248T receptors, and found that they behaved as full agonists in both receptors. This was not absolutely unexpected as α7L248T receptors have been shown to present a rather peculiar pharmacology, capable of being activated by a series of typical nicotinic antagonists such as tubocurarine, dihydro-β-erithroidine, hexamethonium, serotonine, strychnine or bicuculine [14–16,29]. All the latter were also able to activate the doubly mutated α7K145A/L248T receptors. Moreover, changes in the potency of the conventional agonists tested were much larger than those observed in the single mutant *α7K145A*. All these data indicate that the pharmacological phenotype of single mutant *α7K145A* cannot be transferred to α7L248T receptors, probably because the gating mechanisms of α7L248T receptors are deeply altered. Whatever the case, we have also explored the effects of the *K145A* mutation on DMPP-activated single channel currents. Although open channel block cannot be ruled out (DMPP is used at saturating concentrations), single channel currents activated by DMPP greatly differed from those by ACh. Openings are much shorter, gating constants, β/α, are much smaller and several subconductance states might significantly contribute to the overall current. Nevertheless, it should be pointed out that single channel analysis was performed only on the higher conductance openings. This bias and the one originated by the method of selecting the bursts of activity, which excludes very long closures (more frequent with ACh), could partially explain that the quantitative agreement with macroscopic currents data is not complete. However, the effect of substituting Lys145 by Ala is roughly similar to that obtained by ACh: i.e.: closings are longer and openings are slightly shorter in α7K145A/L248T receptors, both resulting in a reduced open probability.

At the single channel level the present work has addressed the mechanism by which Lys145 is involved in the gating of neuronal-type homomeric α7 nAChRs [12] by using the non-desensitizing α7L248T receptor as control. In spite of some quantitative differences, results are similar to those obtained in muscle-type nAChRs, showing that Lys145 is involved in the early steps of channel activation, and suggesting again that the coupling between agonist binding and gating could depend on the type of nAChR studied. Nevertheless, the otherwise deeply altered gating of α7L248T receptors precludes the direct extrapolation of pharmacological data obtained in singly mutated α7 receptors.

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